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# Ddi1-like proteins: a novel family of retroviral-like aspartyl proteases

Proteiny Ddi1: nová rodina retrovirům podobných aspartátových proteas

Bakalářská práce

Školitel: Mgr. Klára Grantz Šašková, Ph.D.

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V Praze

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# Abstrakt

Ubikvitin-proteasomový systém je jedním z klíčových procesů podílejících se na udržení homeostáze buňky. Jeho hlavním úkolem je degradovat poškozené, špatně sbalené či nepotřebné proteiny. Podílí se ale také na celé řadě dalších procesů jako jsou například opravy DNA, regulace buněčného cyklu či signalizace. Celý systém se skládá z mnoha komponent, které jsou vzájemně úzce regulovány. Jednou ze skupin účastnících se ubikvitin-proteasomového systému jsou tzv. proteasomové adaptorové proteiny. Ty rozpoznávají substráty určené k degradaci a transportují je k proteasomu – místu degradace. Mezi tyto proteiny se řadí i rodina Ddi1 podobných proteinů (z angl. DNA damage-inducible protein 1), která se vyznačuje doménou podobnou retrovirovým aspartátovým proteasam. Biologické funkce této proteinové rodiny jsou objasněny jen částečně, a to zejména u Ddi1 pocházející z kvasinky *Sacharomyces cerevisiae*, a jsou proto předmětem aktivního výzkumu.

Tato práce shrnuje dosavadní poznatky a publikované informace o této proteinové rodině, popisuje její obecné charakteristiky, známé funkce, zasazuje je do kontextu buněčných procesů a tím poukazuje na další možné směry výzkumu.



Klíčová slova: ubikvitin-proteasomový systém, ubikvitin, proteasa, poškození DNA

# Abstract

Ubiquitin-proteasome system is one of the key pathways which maintain cell homeostasis. Its purpose is to degrade damaged, misfolded or unnecessary proteins. It is also involved in multiple other processes such as DNA damage repair, cell cycle control or signaling. The entire system consists of multiple components, which are mutually strictly regulated. Important part of this system is group of so called proteasome adaptor proteins. Their role is to recognize and bind targeted substrates and transport them to the proteasome for degradation. Ddi1-like (abbrev. from DNA damage-inducible protein 1) protein family, a group of proteins with retroviral aspartyl protease-like domain, belongs to proteasome adaptor proteins. Global biological role of this protein family is only partially understood the most studied member is Ddi1 protein from *Saccharomyces cerevisiae*, and it is thus a subject of active research.

This thesis summarizes published information about this protein family, describes its general characteristics and known functions, situates them in the context of cell processes and thereby might suggest the course of further study.



Keywords: ubiquitin-proteasome system, ubiquitin, protease, DNA damage

# List of abbreviations

| | |
|---|---|
| AMP | Adenosine monophosphate |
| AMPKs | AMP-activated protein kinases |
| APC | Anaphase-promoting complex |
| ATP | Adenosine triphosphate |
| Ddi1 | DNA-damage inducible protein homolog 1 |
| DUBs | Deubiquitinases |
| ER | Endoplasmic reticulum |
| ERAD | Endoplasmic reticulum-associated degradation |
| FDA | Food and drug administration |
| HDD | Helical domain of Ddi1-like proteins |
| HECT | Homologous to E6AP carboxy terminal |
| hSASP | Human skin aspartyl protease |
| JAMMs | JAB1/MPN/MOV3 metalloenzymes |
| mSASP | Mouse skin aspartyl protease |
| NER2 | Nucleotide excision repair complex |
| NF-κB | Nuclear factor-kappa B |
| NIX1 | Neuronal-interacting factor X 1 |
| Nrf1/ NFE2L1 | Nuclear factor erythroid 2-related factor 1 |
| OTUs | Ovarian tumor proteases |
| PPi | Pyrophosphate |
| RING | Really interesting new gene |
| Rpn | Regulatory particle non-ATPase |
| Rpt | Regulatory particle |
| RVP | Retroviral protease-like domain |
| UBA | Ubiquitin-associated domain |
| UBL | Ubiquitin-like domain |
| UDPs | Ubiquitin-like domain proteins |
| UCHs | Ubiquitin C-terminal hydrolases |
| UIM | Ubiquitin interacting motif |
| UPS | Ubiquitin-proteasome system |
| USPs | Ubiquitin-specific proteases |
| Vsm1 | V-SNARE master 1 |
| XPC | *Xeroderma pigmentosum* type C |

# Table of Contents

# 1. Ubiquitin-proteasome pathway

## 1.1. Basic components of ubiquitin-proteasome pathway

### 1.1.1. Introduction

Cellular homeostasis that keeps the cell alive and functioning, is a strictly controlled state maintained by many complex and regulated processes (1). One of them is proteolysis, a process of degrading either damaged or currently unnecessary proteins, for example many regulatory or signaling proteins (2,3).

There are two ways how to degrade a protein into single amino acids, either specific or non-specific. Cells use non-specific proteolysis to degrade exogenous matter that is used as a source of energy and material for intracellular synthesis. Non-specific proteolysis takes places in lysosomes, small membrane organelles specialized for degradation of endocytosed macromolecules (4). During endocytosis, cytoplasmic membrane forms tiny vesicles, which envelop various cargos from outside the cell and carry it further inside, where they usually fuse with lysosomes. Lysosomal hydrolases, present in the organelle, mostly cleave endocytosed proteins non-specifically. In some cases, however, a specific proteolysis could occur. The non-specific reaction is exergonic and runs at the same pace for any protein (5,6). Lysosomes can digest exogenous or endogenous proteins as well as whole organelles at the end of a process called autophagy. This usually occurs when the cell is in dire need of material or needs to degrade its organelles in initial step of a programmed cell death (7,8).

In a detailed study of proteolysis of endogenous macromolecules, Ciechanover and colleagues (9) discovered yet another, specific and regulated way to degrade proteins that requires energy in the form of ATP. They noticed that in cytoplasm, the proteases and their substrates coexist in direct proximity, yet each protein has a different half-life. This suggests existence of a highly-regulated system which ensures, that only specific substrates are degraded under precisely defined conditions.

This system is nowadays known as the **U**biquitin-**P**roteasome **S**ystem (abbrev. UPS) (10). Aaron Ciechanover, Avram Hershko and Irwin Rose got awarded The Nobel Prize in Chemistry in 2004 for „the discovery of ubiquitin-mediated protein degradation". Proteasome, the central part of this system, is a huge cytoplasmic multi-protein complex, which cleaves proteins into short peptides. The substrates are specifically marked by a small

signaling protein called ubiquitin and subsequently transported to the catalytic site of the proteasome. Ubiquitin is covalently bound to a target protein and forms a polyubiquitin chain with the help of specialized enzymes (11). Those are ubiquitin activating enzyme, ubiquitin conjugating enzymes and ubiquitin ligases. Ubiquitination is reversible due to the activity of deubiquitinases that are able to remove ubiquitin from a substrate and also cleave a polyubiquitin chain (12–14). There are multiple types of ubiquitin chains – each determines the fate of a substrate. For example the most common polyubiquitin chains connected via Lys48 destine the target protein to be degraded in the proteasome (15,16). Other types of ubiquitin linkage have different purpose and will be discussed in the following chapter.

A proper function of UPS is supported by multiple other proteins and enzymes. One of those are proteasome shuttle proteins (or proteasome adaptors), which assist certain substrates in reaching the proteasome in order to be degraded. Ddi1-like protein family is, based on its domain organization, suggested to belong to the family of proteasome shuttle proteins. However, their functions seem to be much more diverse, as further discussed in subsequent chapters.

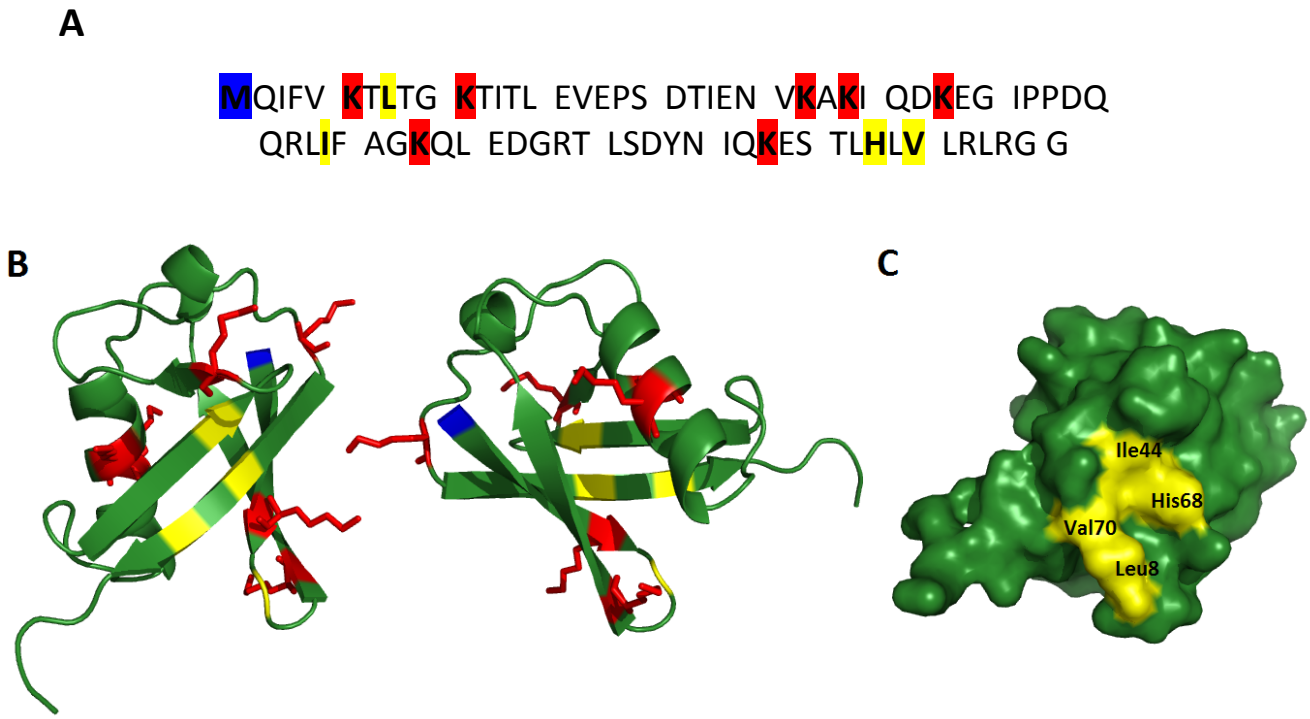


Fig. 1: **Ubiquitin.** (A) Amino acid sequence of ubiquitin with highlighted residues important for interaction: N-terminal methionine (blue), lysines (red) and hydrophobic patch (yellow). (B) 3D structure

of ubiquitin from two different angles with highlighted lysine sided chains (red), N-terminal methionine (blue) and hydrophobic patch (yellow). (C) Surface representation of ubiquitin structure with hydrophobic patch (yellow) that consists of Leu8, Ile44, His68 and Val70 residues. The figure was made with program PyMOL (PyMOL$^{TM}$ v. 1.1) using PDB entry 1UBQ (17).

### 1.1.1. Ubiquitin

Ubiquitin is a small protein consisting of 76 amino acids. It is conserved in all eukaryotic organisms and can be found in every cell in various concentrations (18). As already discussed above, it has an important role in posttranslational modifications of proteins, either in the signaling or in the degradation pathway. In the context of cell function, ubiquitination is very similar to phosphorylation. Both these modifications occur post-translationally, are reversible, need energy in the form of ATP and have various roles within the cell. Phosphorylation mostly occurs on serine, threonine and rarely even tyrosine residue of the target protein, whereas ubiquitination targets multiple lysine residues or even the amino group of the substrate's N-terminus. Phosphorylation utilizes a phosphoryl group ($PO_{4}$-) as a signal, whereas ubiquitination uses a whole protein (19,20). Phosphorylation, unlike ubiquitination also takes part in catabolic reactions of various nutrients beside proteins, namely oxidative phosphorylation (21).

Particular role of ubiquitin depends on the type and site of the ubiquitination performed on individual substrate. Ubiquitination influences localization, interaction properties and activity of many proteins and even processes such as apoptosis or autophagy (22). For example, ubiquitination of histones H2A and H2B seems to be an important part of epigenetics (23). Ubiquitination in yeast and mammals also plays an important role in association of protein complexes that mediate DNA damage repair (24). Further, it plays a significant role in cell cycle progression or endocytosis (25). Taken together, ubiquitin functions as a very versatile and specific signal, due to many possibilities of different ubiquitin linkage (22,26).

The protein itself is coded by four genes in both yeast and human. It is translated either as a fusion protein with a ribosomal subunit (proteins translated from genes *RPS27A* and *UB52*) or it is produced as a polymer precursor (products of *UBB* and *UBC* genes). Both of these forms are later cleaved into separate ubiquitin monomers by specific proteases (27–29).

During the process of ubiquitination, ubiquitin binds to the target protein via an isopeptide bond created on its C-terminal end by a carboxyl group to an amino group on target protein lysine residue (30). A hydrophobic site on ubiquitin formed by Ile44, Leu8, Val70 and His68 amino acid residues is responsible for most of its interactions (31). Its structure and amino acid sequence are shown in *Figure 1*.

Ubiquitin itself has seven lysines in its sequence: Lys6, Lys11, Lys27, Lys29, Lys33, Lys48 and Lys63. Via those lysines and also ubiquitin N-terminal Met1, ubiquitin binds to another ubiquitin and conjugates into chains. The type of lysine bond within the polyubiquitin chain determines its effect on the substrate – each type of chain has a specific role in the cell function (22,26). Various proteins, including proteasome receptors, proteasome adaptor proteins and other ubiquitin-binding proteins, recognize modified substrates and process them further.

Homogenous chains consist of ubiquitins bound to each other via the same lysine residue. The most common within the cell and the most explored are polyubiquitin chains connected via ubiquitin residue Lys48 (hereafter as K48 chain and accordingly with other types of chains) and they function as a signal for degradation (15). Longer K48 chains work as a stronger signal and cause the targeted protein to be degraded faster, whereas homotetramer (or tetraubiquitin) is the shortest chain known to function as a degradation signal (32). As another example of different type of ubiquitin chain that functions as a signal for degradation could serve K11 chain. Inhibition of its formation leads to cell cycle arrest, because K11 chains are responsible for degradation of APC substrates (abbrev. Anaphase Promoting Complex) and thus are important during mitosis. In normal state, APC functions as a E3 ligase, which ubiquitinates its substrates with K11 chains and their degradation enables cell cycle progression (26).

K63 chains are often associated with signaling processes induced by DNA damage (33) or endocytosis (34). They assist during assembly of an enzymatic complex Mms2p/Ubc13p, which is important player in DNA repair in *Saccharomyces cerevisiae* (35,36). K63 chains also play a key role in assembly of complex, which activates transcription factor NF-κB (abbrev. Nuclear Factor-kappa B). Lys63-dependent complex phosphorylates NF-κB inhibitors, causing their degradation and activation of NF-κB, which is important in immune response (37). Polyubiquitination of uracil permease in cytoplasmic membrane of yeast by K63 chain is yet another example of K63 chain function. It induces endocytosis of permease and leads to vacuolar degradation (34).

A non-degradative role was also suggested for K6 polyubiquitin chains. They seem to function in DNA-damage response and replication stress (38,39). Polyubiquitin chains bound through Lys29 and Lys33 also transfer a non-degradative signal. K29 and K33 chains bind protein kinases related to AMPK (abbrev. **AMP**-Activated Protein **K**inase) and negatively regulate their function in an unknown process (40). K27 chains play important role during mitophagy, a process degrading damaged mitochondria (41,42), and therefore deep understanding of their functions is necessary for potential treatment of neurodegenerative diseases such as Parkinson's disease.

Linear polyubiquitin chains are formed by a peptide bond between N-terminal Met1 and C-terminal Gly76 of the other ubiquitin. They function as a precursor for monoubiquitin, as already mentioned above (32). One of the other functions is related to HOIL-1L ubiquitin ligase, which in a complex with HOIP protein assembles polyubiquitin chains (43). This complex further creates linear M1-linked polyubiquitin chains that act as NF-κB activator in similar manner to K63 chains (44).

### 1.1.2. Ubiquitin conjugation cascade enzymes – E1, E2, E3 and E4

The attachment of ubiquitin to the ε-amine of lysine residues of target proteins requires a series of ATP-dependent enzymatic steps catalyzed by E1, which activates ubiquitin; then E2 ubiquitin conjugating enzyme that accepts ubiquitin and passes it to E3 ubiquitin ligase that completes the process by binding ubiquitin to the target protein (26) as shown in *Figure 2*.

Ubiquitin activation by E1 requires energy in the form of ATP. E1 acyl-adenylates C-terminal carboxyl of ubiquitin while catalyzing ATP. In the next step, ubiquitin is bound to a cysteine in the catalytic site of E1, discarding AMP and forming a thioester bond (45). There are two genes *UBA1* and *UBA6* in the human genome capable of catalyzing this initial step, whereas only one gene in yeast (46–50).

Activated ubiquitin is subsequently transferred to E2 enzyme via transthioesterification reaction. Human genome possesses about 35 of E2 enzymes that share highly conserved structure consisting of 150 amino acids known as ubiquitin-conjugating catalytic fold (51).

The final step of the cascade is catalyzed by E3 ubiquitin ligases. They are designated to transfer ubiquitin from E2 to the target protein by creating an isopeptide bond between C-terminal glycine on ubiquitin and a lysine on the substrate. It is also the largest group of ubiquitination enzymes – there are hundreds of them in human genome. The large number of various types of ubiquitin ligases is caused by high demand on substrate specificity (52). There are two families of E3 enzymes transferring ubiquitin in a different way. First of them consists of E3s with HECT domain (abbrev. **H**omologous to **E**6AP **C**arboxy **T**erminal) (53). These E3s first bind ubiquitin via a thioester bond and then directly transfer it to target protein. E3 ligases from the second family include RING domain (abbrev. **R**eally **I**nteresting **N**ew **G**ene), which first binds to the E2 enzyme. Subsequently, the E2-E3 complex cooperates to transfer ubiquitin from E2 to a substrate (54).

There is one more group of proteins from this cascade called U-box proteins, often referred to as E4. These proteins are structurally related to RING ligases but lacking their own specific substrate (55). They assist to E3 ligases in elongation already existing polyubiquitin chains (56).

### 1.1.3. Proteasome

Proteasome, the final component of herein described proteolytic process, is a 2.5 MDa large cylindrical protein complex, consisting of two subunits – 20S core particle (CP) and one or two 19S regulatory particles (RPs), where 19/20S references to their sedimentation coefficient (see *Figure 2*). The core particle, which contains several catalytic sites with various specificity performs the degradation. It is capped on one or both sides by the lid-like regulatory particle (57).

The cylindrical core particle is made of four protein circles, each of them containing seven subunits. Two outer heptameric rings consist of α subunits, which create a narrow entryway with their N-terminal domains and thus allow only partially or entirely unfolded proteins to enter the catalytic chamber (58). The two inner rings, containing the degradation motif, are composed of β subunits, where only three of them are catalytically active (59). The active site of these three – β1, β2 and β5 – is in the lumen of 20S CP (60). The purified complex has three catalytic activities: chymotrypsin-like, trypsin-like and peptidylglutamyl-peptide hydrolyzing. Initially, the whole ring is created in a latent form, the active sites hidden

under N-terminal propeptides and their removal exposes a threonine residue which acts as a nucleophile in hydrolysis and is necessary for cleaving the substrate (61). The degraded protein is hydrolysed into small polypeptides sized from 3 to 15 residues. Some of them play a role in the immune system control, when they are used for presentation on the cell surface via major histocompatibility complex class I (62).

The regulatory particle, capping the core on either one or both sides, is around 900 kDa and consists of two subcomplexes – base and lid. They are made of several subunits named Rpt 1-6 (abbrev. **R**egulatory **P**ar**t**icle) which possess ATPase activity to unfold the protein and Rpn 1-13 (abbrev. **R**egulatory **p**article **n**on-ATPase) which have either structural function or a binding site to recognise a substrate. Part of the RP are also deubiquitinases, which remove the polyubiquitin chain from the substrate. All subunits cooperate to find and bind ubiquitinated protein, cleave off the polyubiquitin chain, unfold the substrate and transfer it to the catalytic site of the core particle (63,64).

Rpn1 has several binding sites and interacts with both polyubiquitin chains and proteins containing a UBL domain (65). One site on Rpn1 can bind a deubiquitinating enzyme, Ubp6, which is able to cleave polyubiquitin chains from substrate and recycle them into monomers. Ubp6 cleaves preferentially ubiquitin from substrates delivered by adaptor proteins, due to its location near the shuttle protein-recognition site (66).

As well as Rpn1, subunits Rpn2, Dss1, Rpn10 and Rpn13 bind polyubiquitin chains (67–69). In human, even homolog of Rpn10, S5a, was proven to bind UBL motives in proteins, which were not meant to be degraded (70). Rpn10 seems to have an additional regulatory function, since it is present in the cell not only as a part of the proteasome but also freely in the cytoplasm. Free Rpn10 binds ubiquitinated substrates and a variety of other proteins, thus it prevents binding of these to proteasome and inhibits their degradation (71,72). This regulation can be hindered by monoubiquitination of Rpn10 particle (73).

Proteasome is a crucial component of intracellular homeostatic processes and thus it is one of the main targets of cancer treatment. First proteasomal therapeutic inhibitor used in humans is bortezomib, approved by FDA for treating relapsed multiple myeloma and mantle cell lymphoma (74,75). Proteasomal inhibition enables/supports correct function of innate immunity (76).

### 1.1.1. Role of deubiquitinases in ubiquitin-proteasome system

Deubiquitinases (DUBs) are a group of specialised isopeptidases, which cleave ubiquitin from a substrate or a polyubiquitin chain. During cleavage of a polyubiquitin chain, the distal ubiquitin is cleaved from the C-terminus of the proximal ubiquitin. Their activity is antagonistic to the activity of E3 ligase. DUBs have multiple roles, for example regulating the amount of free ubiquitin monomers in the cell, editing of polyubiquitin chain or preventing ubiquitin degradation in the proteasome along with the attached substrate (12).

There are five main families of DUBs based on their domain organization and primary sequence: JAMMs (from **JA**B1/**M**PN/**M**OV3 metalloenzymes), MJD class (**M**achado-**J**oseph **D**isease), OTUs (abbrev. **O**varian **Tu**mour Proteases), UCHs (abbrev. **U**biquitin **C**-terminal **H**ydrolases) and USPs (abbrev. **U**biquitin-**S**pecific **P**roteases) (77). JAMMs, hence their name, show metalloprotease activity, other families function as cysteine proteases (78). By now, there are 79 different DUBs in human genome that were proven to be active but there are over hundred more that are predicted (79).

The wide variety of DUBs suggests that they can be very specific, either for a certain type of ubiquitin bond/linkage (80) or for the substrate. There are three proteasome-associated DUBs which take part in proteasomal degradation. Two of them are bound to proteasome subunits, the third contains a proteasome-interacting motif and is transiently associated to the proteasome. These proteasome-associated DUBs cleave off the ubiquitin chain from a protein and thus prevent degradation of the chain in the proteasome. The polyubiquitin tag is hereby detached and recycled by activity of other DUBs, which cleave individual ubiquitins from each other. Another group of DUBs is chain-type specific, cleaving specific chain whether it is freely in cytoplasm or bound to a protein. Last group are substrate specific DUBs, which cleave any chain bound to a particular substrate (78,81).

Generally speaking, DUBs function mostly as regulators, changing the amount of ubiquitinated proteins in the cell and thereby influencing many intracellular processes and also the speed of protein degradation. Low concentration of free ubiquitin monomers in the cell causes increase in DUBs activity, which in other words results in more frequent release of ubiquitin from substrate-bound chains (82).

### 1.1.1. Ubiquitin interacting proteins

There are multiple types of ubiquitin binding components. Those who participate in UPS are divided into three classes of ubiquitin recognizing receptors. The first class contains proteins, which are intrinsic parts of the proteasome, such as proteasome subunits Rpn1, Rpn10 or Rpn13 (83). Second class consists of proteins that recognise and bind substrate in order to transfer it to the proteasome, so called proteasome adaptor or shuttle proteins. They usually contain a **u**biquitin-**l**ike (UBL) domain on the N-terminus and a **u**biquitin **a**ssociated (UBA) domain on their C-terminus (84). The third class contains Cdc48-based complexes, which take part in endoplasmic reticulum-associated degradation (ERAD) pathway (85). All of them aim to transport the targeted protein to the proteasome, which cleaves the substrate into short peptides as described in detail in previous chapter.

## 1.2. Proteasome shuttle proteins

### 1.2.1. General structure and functions of proteasome shuttle proteins

Apart from direct recognition of ubiquitinated substrates by the regulatory particle of the proteasome, additional pathway is orchestrated by so called proteasome shuttle proteins. They recognize and transport certain ubiquitinated substrates to the proteasome as shown in *Figure 2* (86).

Most proteasome shuttle proteins, also called proteasome adaptor proteins or UBA/UBL proteins, belong to the **u**biquitin-like **d**omain **p**roteins (UDPs). Individual functions of UDPs within the cell might differ and will be discussed further.

Yeast Ddi1, Dsk2 and Rad23 proteins belong to the UBL/UBA family. Their domain architecture is schematically pictured in *Figure 3*. They function as adaptor proteins in the proteasome proteolytic pathway by recognizing ubiquitinated substrate and transferring it to the proteasome (87). Inactivation of their genes causes increased amount of polyubiquitinated substrates within the cell (84,88). However, it was also noticed, that even overexpression of these genes causes increase of polyubiquitinated proteins (84,89). This is most likely caused by competitive inhibition, where adaptor proteins without bound substrate occupy binding sites on the proteasome and thus block it for other adaptors,

including those carrying a polyubiquitinated substrate. The same result was acquired in a situation when only one part of an adaptor, for example only the UBL domain of Rad23, was overexpressed (87).

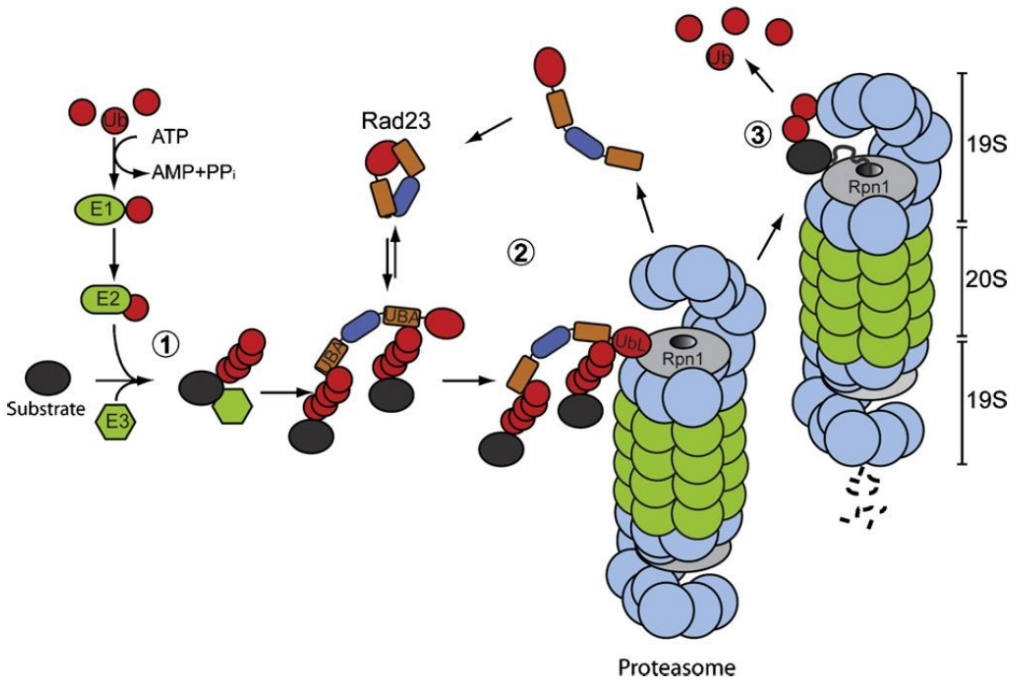


Fig. 2: **The role of proteasome shuttle proteins in the process of ubiquitination followed by proteasomal degradation.** (From left): (1) A target protein (black) is ubiquitinated by E1, E2 and E3 ubiquitin-associated enzymes (green). The newly created polyubiquitin chain functions as a signal for a shuttle protein (in this case Rad23, formed by UBL, Sti1 and two UBA domains), which binds the substrate and transfers it to the proteasome. (2) As soon as the substrate is bound to the Rpn1 subunit of proteasome regulatory particle, the shuttle protein is released and can be reused. (3) Ubiquitin chain is subsequently removed from the substrate and cleaved into monoubiquitins (red) for further utilisation. The substrate is unfolded by the regulatory particle, transferred to the core and eventually cleaved into peptides. Adapted from (90).

The UBA domain of adaptor proteins binds the ubiquitin chain attached to the substrate and probably also protects the shuttle protein from degradation (91,92). These proteins sometimes form dimers or tetramers, because UBA domain is also able to bind some UBL domains (93). UBL domain mimics ubiquitin and is recognised by the proteasome. UBL/UBA proteins contain also other domains significant for their function besides UBL and

UBA domains, for example a Sti1-like domain or a retroviral protease-like domain (RVP) in case of Ddi1 as in detail discussed further (94).

UBA domain, the main effector in substrate binding, forms a compact three-helix bundle. The interaction between ubiquitin chain on substrate and UBA on the adaptor is ensured by hydrophobic sites on helices α1 and α2, which bind Ile44 patch on ubiquitin. UBA domain has higher affinity towards polyubiquitinated proteins or polyubiquitin chains than to monoubiquitin and they are sorted into groups according to their affinity to specific polyubiquitin chains.

Various UBL domains are very similar to each other, but not uniform. They consist of 45-80 amino acids, mostly appear at the N-terminus of a protein and both their sequence and structure resembles ubiquitin (95). They consist of a long α-helix, small $3_{10}$-helix and five antiparallel β-sheets. Interaction with proteasome is ensured via a hydrophobic patch, which is a part of the β-sheet platform exposed on the domain surface (96).

A special type of ubiquitin-binding domain is **u**biquitin **i**nteracting **m**otif (UIM) present in proteasome component S5a, E3 ubiquitin ligases and in a variety of proteins involved in endocytosis, deubiquitination or other cellular processes. UIM consists of 20 amino acids forming an α-helix, which interacts with ubiquitin. To be exact, one side of the motif fits in a hydrophobic patch present on ubiquitin surface. UIM sequence is partially conserved in multiple intracellular proteins and it is not typical for all UBL/UBA proteins. For example, the putative UIM domain of human **D**NA **d**amage **i**nducible protein 2 (Ddi2) could function as a substitute for UBA domain of the adaptor protein as discussed further (97).

### 1.2.2. Dsk2

Dsk2 protein consists of 373 amino acids which form an N-terminal UBL domain, a C-terminal UBA domain and a Sti1-like domain repeat in the centre. Sti1-like motif enables interaction with Stch, protein related to Hsp70 chaperone, due to its homology with chaperone-binding motif of Sti1 phosphoprotein (98). UBA domain of Dsk2 enables the protein to dimerize in the absence of ubiquitin (99).

Yeast Dsk2 and its human homologs were proven to bind proteasome subunits as well as polyubiquitinated chains (100). Moreover, they interact with E3 ligases (101). All these

findings confirm their function as proteasome shuttling proteins (89). Overexpression of Dsk2 in human cells causes accumulation of polyubiquitinated substrates in the cytoplasm inflicted by competitive inhibition of binding sites on the proteasome. Its role is important not only for cytosolic polyubiquitinated substrates, but also for recycling incorrectly folded proteins within endoplasmic reticulum (102). Long term overexpression of Dsk2 causes cell development arrest and eventually cell death (71).

Dsk2 was also shown to take part in mitotic spindle duplication together with Rad23 (103,104). Furthermore, its overexpression in *Xenopus laevis*, where Dsk2 interacts with cyclin A, led to cell division arrest (105).

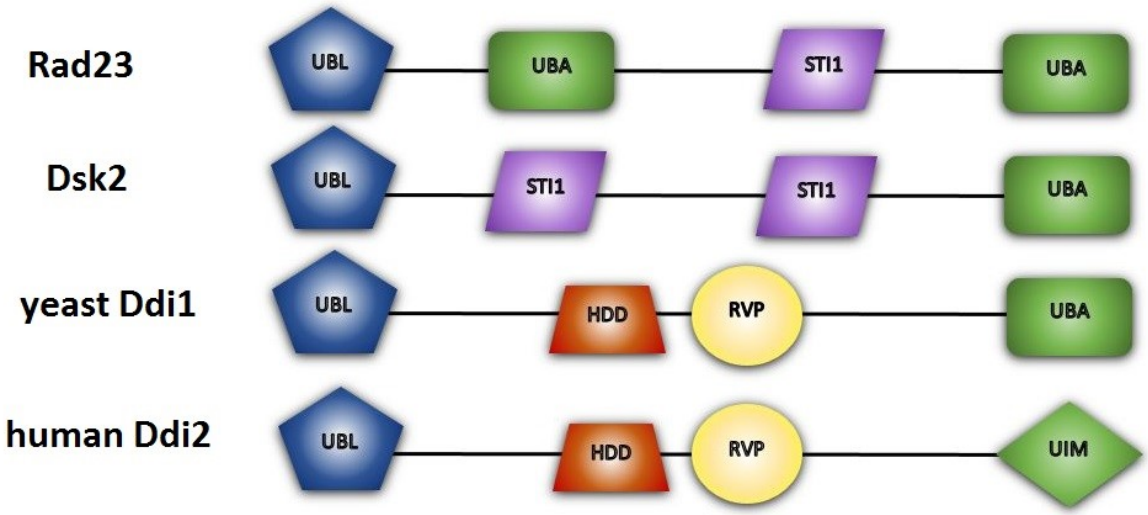


Fig. 3: **Comparison in domain structure organization of proteasome shuttle proteins and members of Ddi1-like protein family.** Descending: Rad23, Dsk2, yeast Ddi1 and human Ddi2. Proportions of proteins and their domains are oversimplified for demonstration.

Dsk2 interacts with the proteasome subunit Rpn10 in the proteasome-bound and also cytoplasmic-free state. Cytoplasmic Rpn10 monomers negatively regulate the amount of free Dsk2 within the cell, preventing it from binding to the proteasome (71). Rpn10 binds a hydrophobic patch on Dsk2 UBL domain in a similar way as it binds ubiquitin (106). Monoubiquitination of Rpn10 lowers its affinity to ubiquitin (73) and Dsk2 and thus increases frequency of Dsk2 - proteasome interaction (107).

### 1.2.3. Rad23

Yeast Rad23 protein consists of 398 amino acids and is highly conserved in eukaryotes from yeast to human (108). Human homolog is biochemically similar to the yeast Rad23 and exists in two variations: hHR23a and hHR23b, which are despite minor differences interchangeable (109). Rad23 is present in both cytoplasm and nucleus (110).

Rad23 has one N-terminal UBL domain, C-terminal UBA domain followed by XPC-binding (abbrev. ***X****eroderma* ***p****igmentosum* type **C**) or Sti1-like domain and the second UBA domain. It was the first described protein to contain a UBL domain and the very first assigned to function as a proteasome shuttle protein (87). Its UBL domain is again very similar to ubiquitin, since it can be substituted with ubiquitin without loss of function of the modified Rad23. However, its deletion causes DNA-damage sensitivity (111).

XPC-binding domain is similar to Sti1-like domain in Dsk2 (112) and its function is crucial for Rad23 role in DNA-damage repair (113). It binds Rpn4 (homolog of human XPC in yeast) creating NER2 complex (abbrev. **N**ucleotide **e**xcision **r**epair) and inducing its connection to UV-damaged DNA. Removing this domain causes increased UV-sensitivity of the cell pointing out its importance in protein function (114,115).

UV-sensitivity can also be caused by deletion of the whole gene or even only of its UBL domain (111). On the other hand, when both UBL domain and XPC domain remain intact and able to perform their role, the UV-sensitivity of cells decreases. Since the UBL domain binds Rpn1 subunit of the proteasome, it suggests that interaction of NER2 complex and the proteasome is crucial in DNA-damage repair. As only proteasome ATPase subunits (present in 19S catalytic subcomplex) take part in this process, it seems that the proteasome plays regulatory and not proteolytic role in this pathway (116,117).

Both UBA domains in Rad23 are able to bind polyubiquitin chains bound via Lys48 (associated with proteolysis) and Lys63 (important for DNA-damage repair) (87,118). UBA domains also function as stabilisation signal and prevent Rad23 from being hydrolysed. In yeast, they enable dimerization of the protein, however human homolog does not dimerize and is mostly present in closed form caused by UBL-UBA interaction. The ability to bind both polyubiquitinated substrates and the proteasome combined with data about proteolysis malfunctions caused by Rad23 overexpression led to first classification of Rad23 as an unbound proteasome subunit (87).

# 2. Ddi1-like proteins

## 2.1. Introduction to Ddi1-like protein family

Ddi1-like (DNA-damage inducible protein 1) proteins belong to UDPs superfamily. The first described homolog was Ddi1 in *Saccharomyces cerevisiae* (119). Ddi1-like proteins are found in all eukaryotes, their structure is highly conserved from yeast to human. Their concentration in cells arises with increased level of damaged DNA, which suggests an important role in DNA repair processes (120).

Like other UDPs, Ddi1-like proteins contain an N-terminal UBL domain and most of them have a C-terminal UBA domain. However, Ddi1-like proteins in vertebrates are missing most of the UBA domain as the C-terminus was shortened in progressive evolution (120). For example, it was recently found out, that UBA was substituted by UIM in human (97).

Ddi1-like proteins have one specific feature, unique among UDPs, a **R**etro**v**iral **P**rotease-like (RVP) domain in the centre of their structure (see *Figure 4*) This domain is named after its structural resemblance to retroviral proteases, which are enzymes forming active dimers responsible for cleaving polyproteins during viral particle maturation (121). The typical sequence for aspartyl proteases (Asp-Ser/Thr-Gly) was shown to be present in RVP domains of Ddi1-like proteins and their ability to form dimers was proven as well (97,122).

Although Ddi1-like proteins are the only group containing RVP domain among UDPs, there are a few other proteins disposing of RVPs in mammals. For example, **m**ouse or **h**uman **S**kin **As**partyl **P**roteases mSASP and hSASP respectively, which play a role in epidermis tissue organisation, or mouse neuron specific nuclear receptor interacting protein (NIX1), which binds ligand-bound nuclear receptors and negatively regulates transcription activation (120,123,124).

Proteolytic activity of the RVP domain was first described in a parasite protozoon, *Leishmania major*. It is active in low pH and it is sensitive to the most common aspartic protease inhibitors (125). Furthermore, enzymatic activity of Ddi1-like proteins was found as well in *Caenorhabditis elegans* (126) and in mammalian cells (127), in context of transcription factor Nrf1 (also known as NFE2L1) activation.

## 2.2. Ddi1 in Yeast

Ddi1 protein in *Saccharomyces cerevisiae* was the first described member of the DNA-damage inducible protein 1 family (Ddi1-like family), named because of its higher transcription rate under genotoxic stress (119). Yeast Ddi1 consists of 428 residues and its structure is formed by an N-terminal UBL domain, HDD (Helical domain of Ddi1) and RVP domains in the middle and a C-terminal UBA domain.

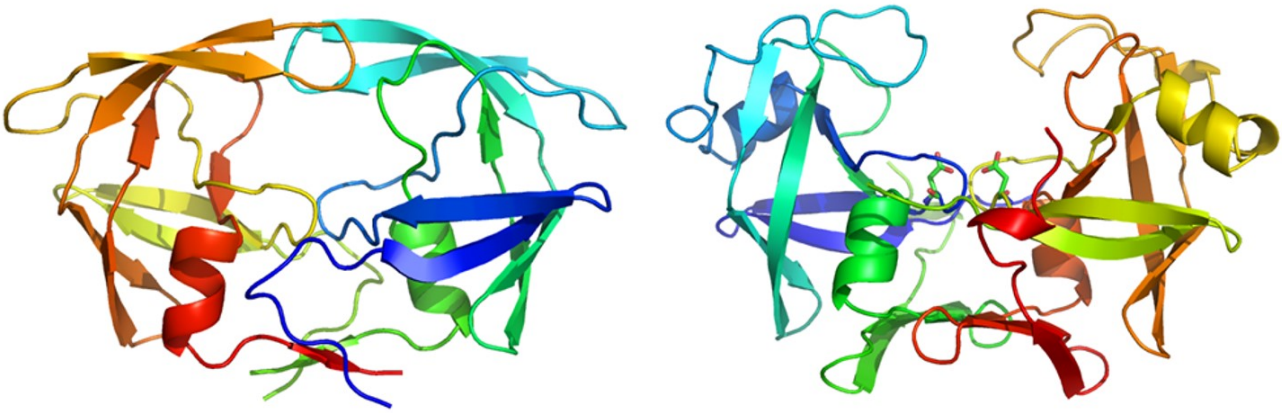

Fig. 4: **Comparison of HIV-1 protease and yeast Ddi1 RVP domain ($Ddi1_{180-325}$).** The fold of RVP dimer of yeast Ddi1 protein (right) resembles structure of HIV-1 aspartic protease (left). The figure was made with program PyMOL (PyMOL™ v. 1.1) using PDB entries 4Z2Z (yeast Ddi1) from (128) and 3GGU (HIV-1 protease) from (129).

Compared to other UDPs, the UBL domain of yeast Ddi1 is quite unique. Despite its low sequence homology to ubiquitin, their structures are quite similar. Unlike in Rad23 or Dsk2, yeast Ddi1 UBL domain does not interact with any ubiquitin binding motif or UBA domain. Surprisingly, it is capable of binding ubiquitin quite strongly – with $K_d \approx 45\text{-}70\ \mu M$. It was therefore suggested, that Ddi1, in a role of a shuttle protein, forms a head-to-tail dimer

and binds the polyubiquitinated substrate with both UBA and UBL domain present on either of the two molecules (93).

Recently, an α-helical domain, between the UBL and the RVP domain, was described. The **H**elical **D**omain of **D**di1-like proteins (HDD) consists of two α-helical regions: the N-terminal region consists of four α-helices and the C-terminal domain forms a hairpin with two α-helices. The sequence of HDD is similar to DNA-binding domain of several bacterial transcriptional regulators. This would suggest that HDD might function as a DNA-binding domain, which is supported by presence of Ddi1 in both cytoplasm and nucleus (128).

RVP domain enables the protein to dimerize, which is most probably crucial for its proper function. In comparison to HIV-1 protease the yeast Ddi1 RVP active site cavity resembles more open conformation as shown in *Figure 4*. Even the inactivated protease domain is able to form dimers (130). The C-terminal UBA domain binds both ubiquitin and Lys48-linked polyubiquitinated substrates and UBL domain interacts with Rpn1 proteasome subunit (108).

Ddi1 protein is most likely a multifunctional protein based on its structural organization. Several biological roles were described for yeast Ddi1 such as cell cycle control or vesicular transport.

Concerning its proteasome shuttle protein function, there are two substrates known for yeast Ddi1 – Ufo1 and HO endonuclease. Ufo1 is an F-box protein which binds HO endonuclease to the SCF complex, and thus enables its ubiquitination and proteasomal degradation (131). The degradation of Ufo1 itself is regulated by Ddi1 which is supported by Ufo1 high level stabilization in *Δddi* mutants. The interaction of Ddi1 with Ufo1 is provided via the substrate UIM domains. Ufo1 is able to autoubiquitinate itself at the four tandem UIM domains, but it needs Ddi1 for the transportation to the proteasome. *Δrad23* and *Δdsk2* single mutants shown no degradation defects for Ufo1, which implies that the bond between Ddi1 ULB domain and Ufo1 UIM domains is specific (132).

The other substrate for yeast Ddi1, HO endonuclease, is a specific DNA cleaving enzyme that enables mating type switching in yeast (133). When HO endonuclease is no longer necessary, it is transported to the cytoplasm, recognised by SCF complex, ubiquitinated and degraded. This pathway makes its half-life within cell only around eight minutes. In case of DNA damage, HO endonuclease is phosphorylated and its degradation rate increases (134). Interaction between Ddi1 and HO endonuclease, as its polyubiquitinated substrate, is mediated by Ddi1 UBA domain and the Ddi1 UBL domain interacts with the 19S

proteasome subunit (131). HO endonuclease is stabilised in *Δddi* mutant cells, which was observed by its more frequent exocytosis in the medium.

Yeast Ddi1 molecule carrying its substrate binds to Rpn1 subunit on the proteasome via a specific interaction with D517 site. Mutations in this region cause accumulation of polyubiquitinated proteins. Substrates of Rad23 or Dsk2 and their degradation rate are not influenced by D517 site mutants (135).

Ddi1 also has a regulatory role in cell cycle progression. Psd1 is a protein that participates on sister chromatid division and once it is ubiquitinated and degraded, transition from metaphase to anaphase occurs (136). Cells with mutated gene *psd1-128* have shown to be temperature sensitive, but overexpression of Ddi1 supresses this effect. It was observed, that Ddi1 protein needs its full UBA domain to be able to rescue *pds1-128* mutant cells (137). It is not clear, how Ddi1 interacts with Psd1, but it was suggested that the process is similar to interaction between Rad23 and Psd1. Rad23 recognises ubiquitinated Psd1, inhibits further ubiquitination and stabilises it (86). These two proteins can substitute each other, since single mutants *Δddi* or *Δrad23* show very little or no defects at all, but a double mutant *Δddi Δrad23* suffers from cell cycle progression defect, mainly during early transition to anaphase, before termination of S1 phase. The double mutant was also highly sensitive to genotoxicity, which supports the hypothesis that both Ddi1 and Rad23 take part in DNA damage repair pathways (137).

Yeast Ddi1 binds v-SNARE and t-SNARE proteins which participate in docking and fusion of vesicles (containing v-SNARE proteins on their surface) and target organelle (containing t-SNARE receptors in its membrane) in both endo- and exocytosis of eukaryotic cells (138). Ddi1 was proven to negatively influence vesicular transport by binding to the SNARE proteins, which are then unable to bind their original interaction partner. There is a 52 residues long sequence between UBA and RVP domain, which ensures interaction of Ddi1 with Sso1 protein. Sso1 binds to Sec9, a t-SNARE protein. This inhibition of Sec9-Sso1 complex assembly causes lower frequency of exocytosis and can be further positively regulated by phosphorylation of autoinhibitory N-terminal domain in Sso1 and also Sso1-binding domain on Ddi1 (130,139). Overexpression of Ddi1 in cells with mutated gene for Sec9 protein causes decrease of the rate of secretion of intracellular proteins in the growth medium or even its entire inhibition. On the other hand, deletion of Ddi1 producing gene causes more frequent secretion, confirming its role as negative regulator of exocytosis (139). It was also suggested that even inactivated RVP domain could play a role in protein secretion repression in cooperation with yeast Ddi1 UBL domain (140).

Ddi1 was earlier addressed as Vsm1 (abbrev **V-S**NARE **M**aster **1**) in context of its role in vesicle transport, where besides Sso1-binding sequence, both RVP and UBL domain play a significant role (140). Other than exocytosis inhibition, Ddi1 is also required in endocytosis of Gα (guanin-nucleotide binding protein) (141).

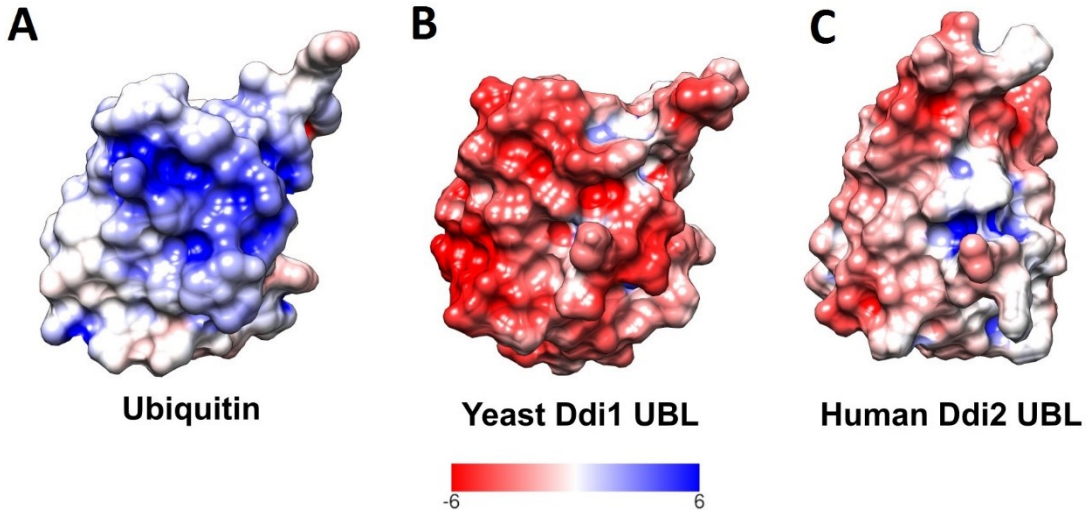


Fig. 5: **Comparison of surface charge of ubiquitin, UBL domain of yeast Ddi1 and UBL domain of human Ddi2.** UBL domain of yeast Ddi1 (B) is noticeably negatively charged, which enables it to bind ubiquitin (A). UBL domain of human Ddi2 (C) has different surface charge than its yeast homolog and therefore binds ubiquitin very weakly.

## 2.3. Ddi1-like proteins in other eukaryotic organisms

Orthologs of Ddi1 protein have been studied in multiple eukaryotic organisms, including *Leishmania major*, *Caenorhabditis elegans*, *Arabidopsis thaliana* and *Drosophila melanogaster*. Ddi1 protein sequence is highly conserved among higher eukaryotes, slightly differing from the one in yeast. The Ddi1-like proteins of *Leishmania* and *C. elegans* were shown to possess only the central catalytic domain, but not UBA, UBL or any other additional domains found in other organisms. Their N-termini contain sequences that are partially similar to the other orthologs, which might mean that the originally present UBL domain was most likely shortened or modified during evolution, maybe entirely deleted (140). The three-domain structure is much more common among higher eukaryotes. Structural organization of

Ddi1 proteins in *Drosophila* and *Arabidopsis* is similar to the one present in yeast – N-terminal UBL domain, C-terminal UBA domain and a domain resembling retroviral proteases in the middle (120).

Recent study of Lehrbach and colleagues describes the catalytic activity of Ddi1 protein in *Caenorhabditis elegans*. Ddi1 activates SKN-1 protein by its cleavage at ER membrane. SKN-1 in *C. elegans* is a homolog of human NRF1 protein, which is required for upregulation of proteasome genes expression (126).

The activity of RVP domain was proven in worm, protozoan and human cells and is assumed for other eukaryotes as well, since the RVP domain sequence is highly conserved.

## 2.4. Human Ddi1 and Ddi2

### 2.4.1. Structure of Ddi1-like proteins in human

There are two genes encoding Ddi1-like proteins in the human genome: *DDI1* located on the chromosome 11 and *DDI2* located on the chromosome 1. *DDI1* gene most likely occurred during evolution through a retrotransposition of *DDI2*. This theory is supported by the fact that *DDI1* contains no introns and *DDI2* is more similar to its orthologs in yeast and non-mammalian vertebrae. The proteins encoded by *DDI1* and *DDI2* genes consist of 396 and 399 residues, respectively. They share around 70% sequence identity and 81% similarity. Both of the proteins are largely understudied.

During evolution of mammals, *RSC1A1* gene was inserted into the sequence of *DDI2* between RVP and UBA domain. The separation of the UBA domain from the rest of *DDI2* sequence caused its inclusion into the *RSC1A1* gene. Ddi2 UBA domain was substituted by a sequence significantly similar to UIM, which in Ddi2 protein binds monoubiquitin with a very low affinity (97).

The Ddi2 UBL domain was also studied for its possible ability to bind ubiquitin as was observed for yeast Ddi1 protein. Despite their fold resemblance, human Ddi2 UBL does not bind ubiquitin. This is caused by different surface charge distribution. The positively charged hydrophobic patch on yeast UBL domain binds the negatively charged ubiquitin quite strongly (93). However, human Ddi2 UBL domain is charged only moderately and interacts with ubiquitin in low millimolar range as shown in *Figure 5*. Human Ddi2 UBL is not able

to bind Ddi2 UIM and so it is not possible for the full length protein to create dimers in a head to tail conformation (97).

The HDD domain found in proximity of central RVP domain is strongly conserved among eukaryotic homologs of Ddi1 and was named because of high abundance of α-helices: **H**elical **D**omain of **D**di2. It consists of four major α-helices which create a hydrophobic core, preceded by one small α-helix. One part of this domain resembles Sti1 domain present in shuttle proteins such as Dsk2 or Rad23. The sequence identity between yeast and human HDD domain is only 25%, which suggests that the human HDD was altered during evolution. This is supported by similar structure of their N-terminal domains' folds, despite their sequence differences (97,128).

The Ddi2 RVP domain forms structure common for retroviral aspartic proteases, which consists of six β-barrels, a β-sheet region which enables dimerization and two helices, which are atypical among retroviral aspartic proteases. Two loops form a flap region also typical for aspartic proteases, with the difference in their extent – the catalytic cavity of Ddi2 is not entirely covered and the two flaps cannot form hydrogen bonds with each other. The catalytic site contains a conserved proteolytic sequence Asp-Ser-Gly-Ala and a water molecule bound between the two Asp (97).

### 2.4.2. Nrf1 and the catalytic activity of human Ddi2 protein

Nrf1, officially named **N**uclear **f**actor **e**rythroid-**2**-**l**ike **1** (NFE2L1), is a transcription factor, which is, under normal conditions, almost instantly degraded after its translation. After transcription, it is translocated to the endoplasmic reticulum (ER), glycosylated and inserted by its N-terminal linker into the ER membrane. A p97/VCP ATPase complex present in the ER membrane almost immediately retrotranslocates Nrf1 into cytosol, where the glycosylation is substituted by ubiquitination and hereby marked protein is degraded in ubiquitin-proteasome proteolytic pathway (142,143).

However, if the proteasome activity is inhibited, Nrf1 is not degraded. Instead, it is cleaved by Ddi2 protein, thus activated and translocated to the nucleus. There it activates transcription of proteasome subunits. Comparison of these two different cell states is shown in *Figure 6*. This so-called bounce-back response causes lower efficiency of bortezomib,

an anticancer drug that targets and inhibits proteasomes as mentioned in Chapter 1.3.3 (144–146).

The function of Ddi2 is thus to cleave and thereby activate Nrf1 and enable its translocation to the nucleus. Mammalian cell depleted of *DDI2* showed lower levels of proteasome production. When these cells were treated with proteasome-arrest drug (bortezomib), accumulation of defective proteins was observed. Ddi2 variants missing proteolytic activity of their RVP domain showed the same phenotype. The activation of Nrf1 in *DDI2* mutants was low but not entirely diminished, which might suggest that there could be yet another enzyme functioning as Ddi2 (127).

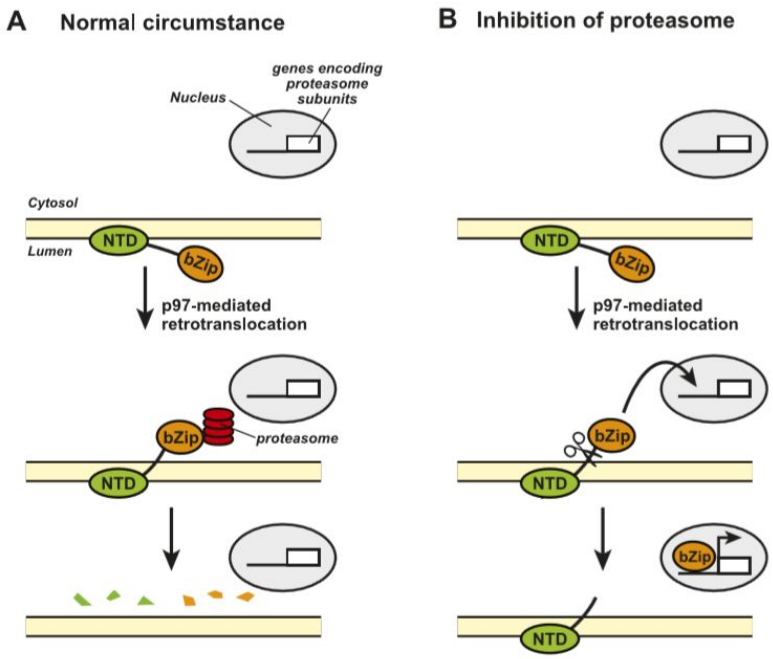


Fig. 6: **Cleavage of Nrf1 by Ddi2.** A) Normal cell state. Nrf1 (consisting of intermembrane N-terminal domain (NTD) linked to the C-terminal basic leucine zipper (bZip) domain) is retrotranslocated by p97 from ER lumen into cytosol in normally functioning cell. Here, the bZip domain is ubiquitinated and degraded by the proteasome. B) In case of proteasome inhibition, Nrf1 is not degraded, but cleaved by Ddi2 (black scissors), activated and translocated to the cell nucleus where it upregulates production of new proteasome components. Adapted from (147).

# 3. Conclusion

Ubiquitin-proteasome system is one of the most important pathways within the cell that helps to maintain cellular homeostasis. It breaks down damaged or unnecessary proteins into peptides that subsequently serve as a material for production of new proteins. Because of its significance, UPS has been thoroughly studied and characterised. However, given its complexity, many processes are still not entirely clear.

Proteasome shuttle proteins are part of UPS. They assist ubiquitinated proteins to reach the proteasome in order to be degraded. They recognise a specific mark – a polyubiquitin chain bound to the substrate by E3 ubiquitin ligase and other ubiquitination cascade enzymes. Shuttle proteins can function specifically for a certain substrate or a certain type of ubiquitination. They possess a UBA domain which binds the ubiquitin chain on a substrate and a UBL domain, which mediates the shuttle protein-proteasome interaction.

Ddi1-like proteins, a group of proteins that belong to the shuttle protein family, are highly conserved in all eukaryotes from yeast to human. Ddi1-like proteins seem to be versatile in their function within the cell. Some of their functions have been already described, such as Ddi1 from *Saccharomyces cerevisiae*, which plays a key role in cell cycle-progression and vesicular transport. Nevertheless, they have been largely understudied.

The role of Ddi1-like proteins in higher eukaryotes was completely elusive until recent revelation of Ddi1 proteolytic activity in worm and Ddi2 in human. It was shown that they regulate transcription of proteasome subunits as a bounce-back response to proteasomal inhibition. Since some cancer treatments target proteasomes, the function of Ddi1-like proteins in tumorous cells might lower their efficiency. This suggests that Ddi1-like proteins might be the next target of new or enhanced treatments alongside with proteasome.

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